GROWTH REQUIREMENTS OF INFLUENZA BACILLI

By T. M. Rivers and A. K. Poole

(From the Department of Pathology and Bacteriology, The Johns Hopkins University)

[From The Johns Hopkins Hospital Bulletin, Vol. XXXII, No. 364, June, 1921]

201 — 204

GROWTH REQUIREMENTS OF INFLUENZA BACILLI

By T. M. Rivers and A. K. Poole

(From the Department of Pathology and Bacteriology, The Johns Hopkins University)

Pfeiffer [1] discovered a new group of bacilli; he said that [202] they were hemoglobinophilic and the cause of influenza. Since then much has been done to prove or disprove the various claims made for these bacilli by their discoverer. The controversy over the etiology of influenza will not be discussed here. Some, however, of the most significant work upon the hemoglobinophilic qualities of these bacilli will be cited and further evidence obtained in this laboratory bearing upon the subject will be submitted in this paper.

Grassberger [2] could not obtain a growth of influenza bacilli on media that contained hematin instead of hemoglobin except in symbiosis with other bacteria. Cantani [3] claimed he could grow them on media enriched with spermatic fluid which did not give the spectroscopic bands of hemoglobin. Ghon and Preyss [4] considered hemoglobin necessary even though it were present in such small quantities that it failed to give the bands with the spectroscope unless hydrazin were added. Nevertheless, they grew influenza bacilli on media containing hematin in symbiosis with other bacteria. Neisser [5] was able to grow influenza bacilli, isolated from a case of purulent conjunctivitis, on nutrient agar for 20 generations in symbiosis with a xerosis bacillus. Rivers [6] reported that he could grow both the hemolytic and non-hemolytic forms of these bacilli for a number of generations on hemoglobin-free media in symbiosis with other bacteria. Putnam and Gay [7] were unable to confirm Neisser's work. Davis [8] thought that hemoglobin acting as a catalytic agent was necessary for growth, and showed that a very small amount was required (1 part in 180,000 parts of medium). According to him, growth will take place in the

[202] presence of coagulated hemoglobin, but if the hemoglobin be broken up by excessive heating into hematin and globin no growth occurs. Fleming [9] was the first to offer proof that seriously interfered with the generally accepted idea, that hemoglobin is essential for the growth of influenza bacilli, by securing a good growth upon agar to which had been added a small quantity of fluid obtained by digesting blood with an equal amount of normal sulphuric acid and then neutralizing this with normal caustic soda. Olsen [10] obtained a growth of influenza bacilli on media containing hematin or hemin in symbiosis with other bacteria. Fildes [11] states that hemoglobin, unless changed, actually inhibits the growth of influenza bacilli and that there are two substances essential for the growth of these organisms. He is probably correct in saying that there are two factors, but his explanation based on the oxygen requirements of the bacilli is not necessarily true. Thjotta and Avery [12] consider two substances as essential for the growth of these bacilli. Both are in blood, one is resistent to excessive heating, the other is not. They do not think hemoglobin as hemoglobin to be one of the factors and also find the heat-labile substance in a number of bacteria and vegetables.

For several years to one of the authors (Rivers) the phenomenon of augmented growth of influenza bacilli in the vicinity of certain other bacteria under various conditions, called symbiosis, has been very interesting and seemed to hold in some way the secret of the real growth requirements of these bacilli. A study of this phenomenon of symbiosis has answered many questions and has shown that influenza bacilli are probably not hemoglobinophilic.

Augmented growth of influenza bacilli in the vicinity of other bacteria is more marked on human or hen blood agar plates than on rabbit, cat, dog or pigeon blood agar plates. In 1919 Rivers [13] showed that on 5 per cent fresh human blood agar a poor growth of influenza bacilli occurred, whereas on 5 per cent fresh rabbit or cat blood agar an abundant growth was obtained. An inhibitory substance, which did not exist in fresh rabbit serum, was found in fresh human serum, was removed by heating the serum for half an hour at 56° C., and

was restored by adding a small quantity of fresh rabbit serum [202]
which in itself was not inhibitory. The phenomenon of symbiosis was marked on 5 per cent fresh human blood agar, whereas this was not true when rabbit or cat blood was used. The addition of the human blood to hot agar (95° C.) destroyed the inhibitory substance, allowing a splendid growth, and the picture of symbiosis sank into the background. Since then hen blood has been found to act similarly to human blood, while dog and pigeon bloods are like cat and rabbit bloods. The explanation for this phenomenon on fresh human blood agar and not on fresh rabbit blood agar was that the symbiotic bacteria removed in some way the inhibitory substance, just as heat does, allowing the influenza bacilli to grow best in their vicinity. This is only a partial explanation for the phenomenon as it occurs on blood agar and does not explain at all the fact that influenza bacilli can be grown on meat-infusion agar in symbiosis with other bacteria.

The change in hydrogen-ion concentration of the medium in the vicinity of the symbiotic bacteria next suggested itself as one of the factors entering into the picture observed on blood agar or meat-infusion agar plates. By some it is contended that this explains fully the augmented growth of influenza bacilli when grown with other organisms. To meat-infusion agar, pH 6.8, 7.6 and 8.6, was added 2.5 per cent fresh rabbit blood and 1 per cent glucose. Plates were made and inoculated with influenza bacilli. In the center of the plates, the three hydrogen-ion concentrations being used for each organism, were streaked *B. coli, B. alkaligenes,* and *Staphylococcus aureus.* The staphylococci and colon bacilli fermented the glucose and inhibited completely the growth of influenza bacilli near them on the plates with pH 6.8 and 7.6 (hydrogen-ion determinations were made before the blood was added). On the most alkaline plate, pH 8.6, the growth was best near the streak of colon bacilli, probably because of a change in the hydrogen-ion concentration to a more favorable [203]
one for the growth of influenza bacilli. The opposite occurred on the plates streaked with *B. alkaligenes.* The growth of influenza bacilli was best near the streak of *B. alkaligenes* on the plates with pH 6.8 and a complete inhibition of growth

[203] occurred near the streak on the plate with pH 8.6. The blood near the center streaks was hemolysed and it could be seen that the hydrogen-ion concentration had been changed when the plates were flooded with brom-cresol purple. The striking thing, however, was that often just outside the zone of inhibition another zone of remarkably augmented growth occurred, reading thus: Streak of staphylococci near which would be no growth of influenza bacilli, then an unusually good growth, and then the usual growth on the plate at that hydrogen-ion concentration. Some of the phenomenal growth just beyond the zone of inhibition probably can be explained on the supposition that the hydrogen-ion concentration at that point and the action of the acid or alkali on the blood produce something more suitable for the use of influenza bacilli or liberate something in the process of breaking up the blood cells. This will be spoken of later.

Alkalies and acids were used for the center streaks to see if they would give the same results as bacteria. Blood agar plates of different hydrogen-ion concentrations were made as above and inoculated with influenza bacilli. Then acid or alkaline agar at 45° C. was placed on the center of the plates. Near these streaks the blood was hemolyzed or made brown and the same picture of inhibition and augmentation in the growth of the influenza bacilli was obtained as when bacteria were used, except that outside the zones of inhibition the areas of augmented growth, although present, were not so marked.

The fact that the zones of augmentation were more marked when bacteria were used suggested that they did not occur solely from an alteration in the blood, from the liberation of some substance in the blood cells, or from a change in the hydrogen-ion concentration, but in some way were influenced by the symbiotic bacteria. This is further confirmed by the fact that in growing influenza bacilli on meat-infusion agar in symbiosis with other bacteria the growth always occurred in the influenzal transplant nearest the symbiotic bacterial transplant. Sometimes the two transplants were half an inch to an inch apart. Whatever this substance may be, it is diffusible and reached the influenza bacilli by diffusion through the medium rather than by being carried to them by way of the

air. This diffusible substance was considered one of the [203]
factors producing augmentation of growth of influenza bacilli near other bacteria and possibly a necessary factor for the growth of these bacilli under all conditions.

As it seemed possible that, in different bacteria or liberated by them in the process of growth, there is something which will augment the growth of influenza bacilli under various conditions, we added the extracts of bacteria to the media instead of making symbiotic cultures. Yeast was used first because it could be obtained easily in large quantities. One cake of bakers' yeast was mixed with 50 c. c. of distilled water, boiled one minute, filtered, and sterilized through a Mandler filter. To 100 c. c. of meat-infusion broth 15-20 c. c. of this yeast extract was added. This mixture was tubed in 10 c. c. quantities and was incubated to be sure of its sterility. When this medium was inoculated with influenza bacilli a good growth was obtained. The cultures were carried for 10 generations and discontinued. The first tube was inoculated with a loop of bacteria from a blood agar slant. Subsequent transplants were made by using 0.5 c. c. of the broth cultures. The bacilli in the tenth generation were growing normally, looked as usual under the microscope, formed indole, and were in pure culture when plated. When autoclaved yeast extract was used instead of that sterilized through a Mandler filter the medium was found unsuitable for the growth of influenza bacilli. The meat-infusion broth was made in the usual way and had been autoclaved 15 minutes under 15 pounds of pressure. These facts indicate that in yeast there is something essential for the growth of influenza bacilli and that it can be destroyed in the autoclave under 15 pounds of pressure for 15 minutes.

At this point the problem seemed an easy one, but soon it was observed that only certain lots of meat-infusion broth supported growth of influenza bacilli for many generations when the unautoclaved yeast extract was added. Another substance also appeared necessary for their growth and sometimes it was present in meat-infusion broth, at other times absent. Interest was fixed upon this second factor in attempts to find out what it was.

[203] In looking over some experiments, several interesting facts were observed. Agar made with 2 per cent peptone and 0.5 per cent sodium chloride permitted growth of influenza bacilli only when more than 2.5 per cent fresh rabbit blood was added while the agar was at 45° C., and yet allowed a good growth when 0.5 per cent blood was added while the agar was at 95° C. One drop of blood added to 100 c. c. of meat-infusion agar at 45° C. permitted little or no growth of the bacilli. If the agar were at 95° C. when the drop of blood was added a fairly good growth occurred. This was striking. So little blood, one drop in 100 c. c. of medium, certainly was not inhibitory because of some deleterious substance, inasmuch as 5 per cent of the same fresh blood with the same kind of agar allowed an abundant growth. Apparently in 5 per cent fresh unheated rabbit blood there were free enough growth-producing substances; while in one drop of unheated blood in 100 c. c. of agar not enough was supplied. Heating this one drop of blood to 95° C made available sufficient growth-producing substances. Others find that this alteration in the blood can be made with acids or pepsin as well as by heat.

The autoclave stable factor which at times was present in meat-infusion broth was sought for in blood. A clot from 500 c. c. of blood was infused with 200 c. c. of physiological saline solution, boiled, filtered, and autoclaved half an hour under 15 pounds of pressure. This autoclaved extract of blood clot was slightly brownish but did not give the spectroscopic bands of hemoglobin. An extract of yeast was prepared, half of which was sterilized through a Mandler filter, the other half in the autoclave. Two per cent peptone water
[204] or agar was used as the basic medium. Influenza bacilli did not grow on these alone. If enough unheated rabbit blood, or "chocolate blood" were added, a good growth occurred. If 15-20 c. c. of the autoclaved blood clot extract, the filter-sterilized yeast extract, or the autoclaved yeast extract, were added alone to the peptone water or agar, no growth was obtained. If the autoclaved blood clot extract and the autoclaved yeast extract were added, there was no growth. If, however, the filter-sterilized yeast extract was used with the

autoclaved blood clot extract, an abundant growth was secured for as many generations as desired. [204]

When these facts are analysed, two substances at least are shown to be essential for the growth of influenza bacilli. Both of these are in blood; one resists autoclaving for 30 minutes under 15 pounds pressure, the other does not. The autoclave labile factor is found also in yeast. The autoclave stable one is not hemoglobin, if the spectroscopic examination or if autoclaving the blood clot extract half an hour be proof enough. So far this last factor has not been found outside of blood and may come from blood pigment even though it is not hemoglobin itself. In making media it has been noticed that some meats are bloodier than others. Certain lots of meat-infusion broth must contain enough of the autoclave stable substance from blood, as the growth of influenza bacilli is supported when unautoclaved sterile yeast extract is added. This explains the phenomenon of symbiosis on meat-infusion agar. The autoclave stable substance from the blood is in the agar and the autoclave labile factor is furnished by the symbiotic bacteria.

CONCLUSIONS

1. The phenomenon of augmented growth of influenza bacilli in the vicinity of other bacteria on solid media may be due to any one or, at times, all of the following factors:

(a) The removal of inhibitory substances that are marked in certain bloods, as human and hen's blood.

(b) The change of the hydrogen-ion concentration to one more favorable for the growth of influenza bacilli.

(c) The alteration in the blood, making growth substances more available.

(d) The production by the symbiotic bacteria of an autoclave labile substance necessary for the growth of influenza bacilli.

2. Two substances are essential for the growth of influenza bacilli. Both are in blood. One resists autoclaving half an hour under 15 pounds pressure, the other does not. The autoclave stable substance is not hemoglobin, although it may be derived from the blood pigment, and as yet has not been found

[204] outside of blood. The autoclave labile substance has been obtained also from yeast.

3. In what way these two factors operate to promote the growth of influenza bacilli is not known.

BIBLIOGRAPHY

1. Pfeiffer, R.: Ztschr. f. Hyg., 1893, XIII, 357.
2. Grassberger, R.: Cntrlbl. f. Bakteriol., 1898, XXIII, 353.
3. Cantani, A.: Ztschr. f. Hyg., 1901, XXXVI, 29.
4. Ghon, A., and v. Preyss, W.: Cntrlbl. f. Bakteriol., 1902, I, Orig., XXXII, 90.
5. Neisser, M.: Deutsche med. Wchnschr., 1903, XXIX, 462.
6. Rivers, T. M.: Bull. Johns Hopkins Hosp., 1920, XXXI, 50.
7. Putnam, J. J., and Gay, D. M.: Jour. Med. Research, 1920, XLII, 1.
8. Davis, D. J.: Jour. Infect. Dis., 1907, IV, 73.
9. Fleming, A.: The Lancet, London, 1919, CXCVI, 138.
10. Olsen, O.: Centralbl. f. Bakteriol., 1920, Orig., LXXXV, 12.
11. Fildes, P.: British Jour. Exper. Path., 1921, II, 16.
12. Thjotta, Th., and Avery, O. T.: Proc. Soc. for Exp. Biol. and Med., 1921, XVIII, 197.
13. Rivers, T. M.: Bull. Johns Hopkins Hosp., 1919, XXX, 129.